Supplemental Data

Material and Methods

*(In addition to the methods used in supplemental figures, this part includes detailed information for the methods used in the main paper***)**

Assay for intracellular transport of C5-DMB-Cer- CHO cells on glass-bottom dishes were labeled with 1 μM C5-DMB-Cer complexed with BSA (1) in Nutridoma-supplemented DMEM F12 medium for 30 min at 4°C. After wash, cells were pre-incubated with DMSO (control, 0.1% final concentration), 5 μM CHC or 2.5 μM HPA-12 for 30 min at 4°C. They were then incubated for 30 min at 37°C, washed and fixed with 3% PFA at 4°C. Cells were observed just after fixation with LSM 510 confocal microscope.

Assay for C6-NBD-SM recycling- CHO cells were preincubated for 30 min at 37°C with 5 μM CHC or DMSO (control, 0.1% final concentration) in DMEM F12 without phenol red, and then 4 μM C6-NBD-SM was added for 30 min at 37°C, to allow cell surface labeling and internalization (2-4). After internalization, cells were washed and NBD fluorescence on the cell surface was quenched by 1 min incubation with 50 mM dithionite. Cells were then chased for 30 min at 37°C in DMEM F12 medium in the absence of dithionite and NBD fluorescence was observed with a Zeiss LSM 510 microscope**.**

In vitro assay of CerS2 and CerS5- CerS activity was assayed as described (5,6). Briefly, HEK cells were transfected with human CerS2 or CerS5 using the PEI reagent (Sigma). Cells were collected by centrifugation and homogenized in Hepes-NaOH buffer (pH 7.2). Homogenates (50μg for CerS5 and 100μg for CerS2) were incubated in a final volume of 250 μl Hepes-NaOH buffer with 0.25μCi of [4,5-^{3}H]sphinganine/15 μM sphinganine/20 μM defatted-BSA/50 μM C22-CoA (for CerS2 activity) or C16-CoA (for CerS5 activity) for 20 min at 37°C. Increasing concentrations of CHC were added to the reaction mix prior to addition of substrates. Reactions were terminated by the addition of chloroform:methanol (1:2 by vol) and lipids extracted (7); the organic phase was loaded on TLC and lipids were separated using chloroform:methanol:2 M ammonium hydroxide (40:10:1 by vol/vol) as the developing solvent. Dihydroceramide was identified using authentic standards. ^{3}H-labeled lipids were visualized using a phosphorimaging screen (Fuji, Tokyo, Japan), recovered from TLC plates by scraping the silica directly into scintillation vials, and quantified by liquid scintillation counting.

In vitro enzymatic assay of SM synthase- Membrane fraction of CHO cells used as the SM synthase source were prepared as previously described (8) except that CHO cells grown in 100-mm dishes were disrupted by passage through G27 needle. The postnuclear supernatant was centrifuged at 100,000 x *g* for 1 h and the pelleted membrane fraction was suspended in 10 mM Tris-HCl pH 7.40, 0.25 M sucrose and stored at -80°C until use. Assay of SM synthase activity was performed as described (8,9) with some modifications. C5-DMB-Cer (1 μM), C12-NBD-Cer (1 μM) or *N*-[1-^{14}C]hexanoyl-D-*erythro*-sphingosine (C6-Cer) (2 μM) complexed with BSA was preincubated with CHC (10 and 50 μM) for 10 min at room temperature respectively, in 800 or 400 μl of 10 mM Hepes-NaOH buffer (pH 7.5) containing 2 mM EDTA. Then 400 μg of CHO membrane were added and incubation was performed at 37°C for 15 and 60 min. Lipids were extracted (7) and analyzed by HPTLC as in Fig 2A. Fluorescent spots were quantified with Typhoon 9140 (GE Healthcare) whereas radioactive spots were quantified by BAS 5000 image analyzer.

Analysis of intracellular VSVG protein trafficking from ER to Golgi- HeLa cells were plated and grown for 2 days on glass-bottom dishes (Iwaki, Tokyo, Japan). Then they were transiently transfected for 4 h at 37°C with the plasmid pVSVG-EGFP (a generous gift from Dr Lippincott-Schwartz) using Lipofectamine LTX (InVitrogen, CA) and Opti-MEM medium as indicated by the manufacturer. The pVSVG-EGFP

plasmid codes for the vesicular stomatitis virus G glycoprotein (VSVG) tagged with the enhanced green fluorescent protein EGFP from the temperature sensitive mutant strain ts045 (10,11). Cells were grown overnight in the medium containing 10% FBS at the non-permissive temperature 39.5°C in order to accumulate the VSVG protein in the ER. Cells were then treated with 5 μM CHC for 1 h at 39.5°C in serum-free medium, followed by the incubation at the permissive temperature 32°C for 1 h up to 5 h. At indicated intervals, cells were observed with Zeiss LSM confocal microscope.

Metabolic labeling of cell lipids with [^{14}C]serine and [^{14}C]choline- Subconfluent CHO cells were incubated at 37°C in Nutridoma-supplemented F12 medium (serum-free) in the absence or the presence of limonoids or inhibitors for indicated periods. Then, [^{14}C]serine (1 μCi/ml) was added and the incubation was extended for 2 h. For [^{14}C]serine labeling of BFA-treated cells, subconfluent CHO cells were incubated at 37°C for 1 h in Nutridoma-supplemented medium in the absence or the presence of limonoids or inhibitors, then treated with 1 μg/ml BFA for 30 min. Cells were further labeled for 2 h with [^{14}C]serine. For [^{14}C]choline labeling, CHO cells were incubated in Nutridoma supplemented medium without or with limonoids for 1 h at 37°C. Then [^{14}C]choline (1 μCi/ml) was added and incubation was continued for additional 4 h in the absence or presence of the drugs. At the end of the incubation time, lipids were extracted and analyzed by HPTLC as described below. To study the degradation of [^{14}C]SM, subconfluent CHO cells were labeled for 2 h with [^{14}C]serine. After wash, cells were incubated with Nutridoma-supplemented medium containing 10 mM L-serine at 37°C in the presence or absence of CHC. Cells were harvested at the indicated times.

Study of the conversion of [^{3}H]Cer to [^{3}H]SM in cells- CHO cells were analyzed for the conversion of [^{3}H]Cer to [^{3}H]SM as described previously (1) with some modifications. Cells were washed with cold plain phenol red-free DMEM-F12 medium and pulse-labeled for 30 min at 15°C with [^{3}H]dihydrosphingosine ([^{3}H]DHS) (1 μCi/ml) in DMEM-F12 with 1% Nutridoma. After wash, cells were treated with 5 μM CHC or 1 μM HPA-12 for 30 min at 4°C. Then they were incubated at 37°C for various times in Nutridoma DMEM-F12 after the addition of 40 μM fumonisin B1 (FB1), the ceramide synthase inhibitor (12). Lipids were extracted and analyzed as described below.

Lipid analysis and determination of lipid content- At the end of the incubation time, radiolabeled cells were washed twice with cold PBS and harvested in cold 2 mM EDTA or 0.1% Triton X-100. Cell lysates were aliquoted for protein quantification and extracted by the method of Bligh and Dyer (7). [^{14}C]serine- or [^{3}H]DHS-labeled lipids were separated on HPTLC plates with the solvent mixture methyl acetate: *n*-propanol:chloroform:methanol: 0.25% KCl (25:25:25:10:9, v/v) (1)**.** [^{14}C]choline-labeled lipids were separated on HPTLC plates with solvent mixture chloroform:methanol: water: acetic acid (65:43:3:1, v/v) (13)**.** Radioactive spots were quantified with a BAS 5000 image analyzer (Fuji Film Inc., Tokyo, Japan). To compare samples, arbitrary units (AU) of the radioactivity were divided by the protein content and results expressed as AU per protein or as percentage of the control after normalization to protein.
For the determination of the phospholipid and SM content, cells were incubated in Nutridoma-supplemented medium at 37°C without or with drugs for 2 days. Medium was replaced after 24 h. Then, the cells were harvested and lipids extracted as described above. To evaluate SM content, the total lipid extract was separated on HPTLC plates (13). After visualization by primuline staining, the SM spots were scraped and extracted (7). Total phospholipid content from total lipid extracts and SM content were evaluated by phosphorus quantification (14).

Mass spectrometric analysis of limonoids after incubation- To analyze the pH effect, aliquot of HC (40 nmol) was incubated in 100 μl of methanol:water (1:1, v:v) at pH 7 (neutral) or acidified with HCl 1N to pH 5 and incubated at 37°C. The samples were directly injected in the mass spectrometer. To analyze limonoid after cell incubation, CHO cells were incubated in Nutridoma-supplemented medium at 37°C

with or without HC (10 μM) for 1h and 24h. Then cells were harvested and limonoids extracted by the method of Folch (15). An Agilent 1100 series LC (Agilent Technologies, Santa Clara, CA) coupled to a 4000 QTRAP hybrid triple quadrupole mass spectrometer (AB SCIEX, Foster City, CA) was used to analyze limonoids. The samples were injected onto a reversed phase C18 column (CAPCELL PAK C18 MG III, 2.0 × 50 mm, Shiseido CO., LTD., Tokyo, Japan) at flow rate 0.2 ml/min and the column temperature was kept at 30˚C. Solvent A (0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) were used as eluent. The samples were eluted through the following gradient condition: Solvent A/B (8:2) 0.5 min followed by a linear gradient to A/B (5:5) over 25 min. After 5 min at 50% solvent B, the gradient was change to A/B (1:99) over 5 min and keep for 5 min to wash the column. The gradient was brought back to A/B (8:2) and the column was then equilibrated for 5 min. The optimal conditions for ionization and fragmentation were determined. After the Q1 setting (m/z 485.5, $[M+H]^+$) was optimized, product ion spectra (MS/MS) was collected across a range of collision energies. Structurally specific product ions were identified (m/z 417.5), and collision energy and collision cell exit potential were manipulated to produce optimal signal for the product ion of choice. Multiple reaction monitoring (MRM) was set to positive measurement mode with following instrument parameters: declustering potential of 90, entrance potential of 10, curtain gas of 10, ion spray voltage of 5500, temperature of 400, nebulizer gas of 40, auxiliary gas of 40, collision energy of 25, collision cell exit potential of 10, interface heater was on, and the dwell time was 100 ms. Both Q1 and Q3 were set to unit resolution. Standard curve was established by using standard HC at a concentration of 0-20 pmol/μL and the relative quantification was performed with peak areas observed in MRM. Data acquisition and analysis were performed using Analyst Software version 1.4.1 (AB SCIEX).

Measurement of fluorescence anisotropy of DPH- Lipids (1,2, dipalmitoyl-sn-phosphatidylcholine (DPPC) or the mixture of egg PC (PC): egg PE (PE): C16-Cer (32:8:2 mol/mol) were evaporated under nitrogen and vacuum-dried. Then they were hydrated by adding the buffer HNE to get a final concentration of 1 mM for DPPC and 0.8 mM for PC:PE:Cer vesicles. They were vortexed then sonicated in a bath sonicator. Aliquots (100 μl) of DPPC vesicles were incubated with increasing concentrations of HC from 100:1 to 5: 1 molar ratio for 15 min at 37 ^{o}C. Aliquots (100 μl) of PC:PE:Cer vesicles were incubated with 8 μM HC for 15 min at 37 ^{o}C. Then 0.5 mol% DPH was added to the vesicles from a tetrahydrofuran (THF) stock solution and incubated for 5 min at 37 ^{o}C. In some experiments, HC was directly included during the lipid film preparation. The fluorescence was monitored with a fluorometer JASCO FP-6500 equipped with a computer-controlled polarizer at a scan rate of 2^{o}C/min (from 20 to 60^{o}C) (DPH, λexc= 360 nm and λem= 428 nm). The steady-state anisotropy r was evaluated from the fluorescence intensities as previously (16).

Differential Scanning Calorimetry (DSC)- Stock solutions of DPPC, C16-Cer and limonoid HC were dissolved in chloroform. Lipophilic films were formed after evaporation of lipid solutions with or without limonoid compound under nitrogen gas and dried in high vacuum. They were hydrated and vortexed in 50 mM Hepes-NaOH buffer (pH 7.5) containing 0.5 mM EDTA. The final lipid concentration in the vesicles was 1 mM. DSC was performed on a Microcal VP-DSC microcalorimeter (MicroCal, Northampton, MA). DSC thermograms were recorded at a scan rate of 60 ºC/hr for all samples. DSC scan was performed at least 15 times. The absence of lipid damage after DSC measurement was monitored by TLC after the extraction of lipids. The obtained data were analyzed and plotted with Origin software (OriginLab Corporation, Northampton, MA) as previously described (17).

Other methods- Phospholipid phosphorus was determined as described (14). Protein content was measured by Bradford protein assay (Bio-Rad reagent) or Pierce BCA assay (Piece reagent). Mean and average deviation (AD) were used for two independent experiments. Mean and standard error mean (sem)

or standard deviation (sd) were used for n=3 independent experiments. Statistical analysis was performed according to paired Student's t-Test.

Supplemental Figure Legends

Suppl. Fig. S1: CHC does not inhibit the intracellular traffic of VSV-G protein: HeLa cells were transiently transfected for 4h at 37°C with the plasmid pVSVG-EGFP (from the temperature sensitive mutant strain ts045), then grown overnight at the non-permissive temperature 39.5°C. Cells were then incubated in the absence (control, **A-C**) or the presence of CHC (5 μM, **D-F**) for 1h at 39.5°C (**A, D**). They were further incubated at the permissive temperature 32°C for 1h (**B, E**) up to 5 h (**C, F**). Cells were observed with Zeiss LSM confocal microscope Bar, 10 μm.

Suppl. Fig. S2: CHC does not inhibit CerS and SM synthase activity *in vitro*: CerS2 (**A**) and CerS5 (**B**) activity was examined *in vitro* upon addition of increasing concentrations of CHC. The inserts show CerS activity in the lower concentration range of CHC. Lipids were extracted, analyzed by TLC and radioactive spots were quantified by liquid scintillation counting. **C**) C5-DMB-Cer (1 μM), C12-NBD-Cer (1 μM) or ^{14}C-C6-Cer (2 μM) were preincubated with CHC (10 or 50 μM) for 10 min, then CHO membrane used as SM synthase source was added and incubation performed at 37°C for 15 min (grey bar) and 60 min (dark bar). Lipids were extracted and analyzed by HPTLC. Fluorescent SM spots were quantified with Typhoon 9140 (GE Healthcare) whereas ^{14}C-SM spots were quantified by BAS 5000 image analyzer. Values are expressed in percentage of the control after normalization of the fluorescence intensity or radioactivity by the protein content.

Suppl. Fig. S3: (A) Effect of the limonoids on PS and PE biosynthesis: CHO cells were treated as in Fig 8A. Grey bars: phosphatidylserine (PS) and white bars: phosphatidylethanolamine (PE); **(B)** Lack of the effect of the limonoids on metabolic labeling of phosphatidylcholine (PC): CHO cells were treated with 1 μM (black bars) or 10 μM (grey bars) limonoids for 22 h followed by 4 h labeling with [^{14}C]choline as in Fig 2D. Lipids were extracted and analyzed after separation on HPTLC as described in Material and Methods. Results are expressed in the percentage of the control after normalization of the radioactivity by the protein content. **(C)** Some limonoids did not inhibit SM biosynthesis: CHO cells were preincubated for 22 h with 10 μM limonoids or with 40 μM fumonisin B1 (FB1), then cells were labeled with [^{14}C]serine for 2 h in the presence of the drugs. See Fig 7 for names of compounds. Lipids were extracted and analyzed as in Fig. 2A. Results are expressed as percentage of the control after normalization of the radioactivity by the protein content;

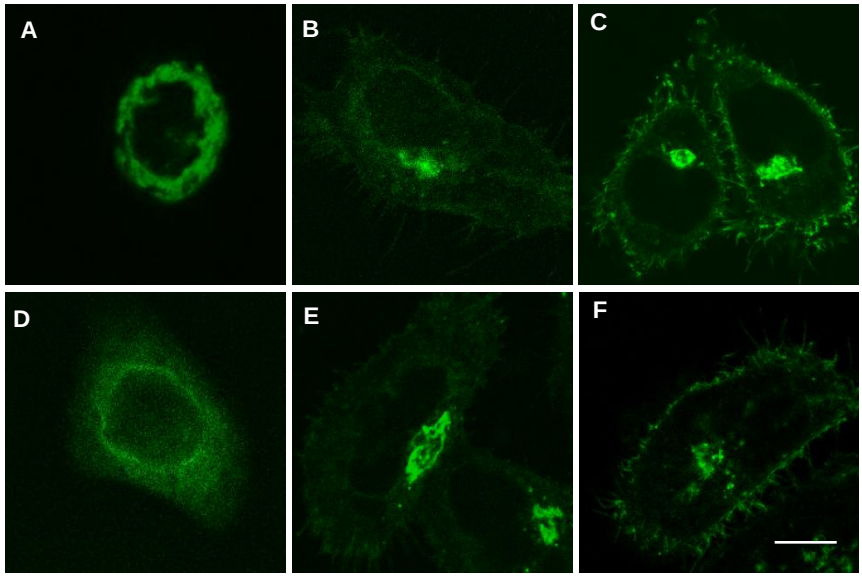
Suppl. Fig. S4: Limonoid HC remained intact after incubation: **(A)** Mass spectra of limonoid HC in the pH range 5-7: HC was preincubated for 24 h in neutral (upper spectrum) or acidic (lower spectrum) conditions then analyzed by MS as described in supplemental Materials and Methods. A major peak was seen at m/z 485 $[M+H]^+$ corresponding to the precursor ion (intact hemiacetal) with a minor peak at m/z 467 corresponding to the fragment without the hydroxyl whose ratio did not change with the pH conditions. **(B)** Limonoid HC remained intact after cell incubation: CHO cells were incubated for 1 h and 24 h with 10 μM HC. Then cells were extracted and analyzed by LCMS-MS with quantification by MRM of the precursor ion m/z 485.5, $[M+H]^+$ as described in Supplemental Materials and Methods. Data are expressed in nmol of HC/mg proteins. They are the mean and SD of triplicate samples.

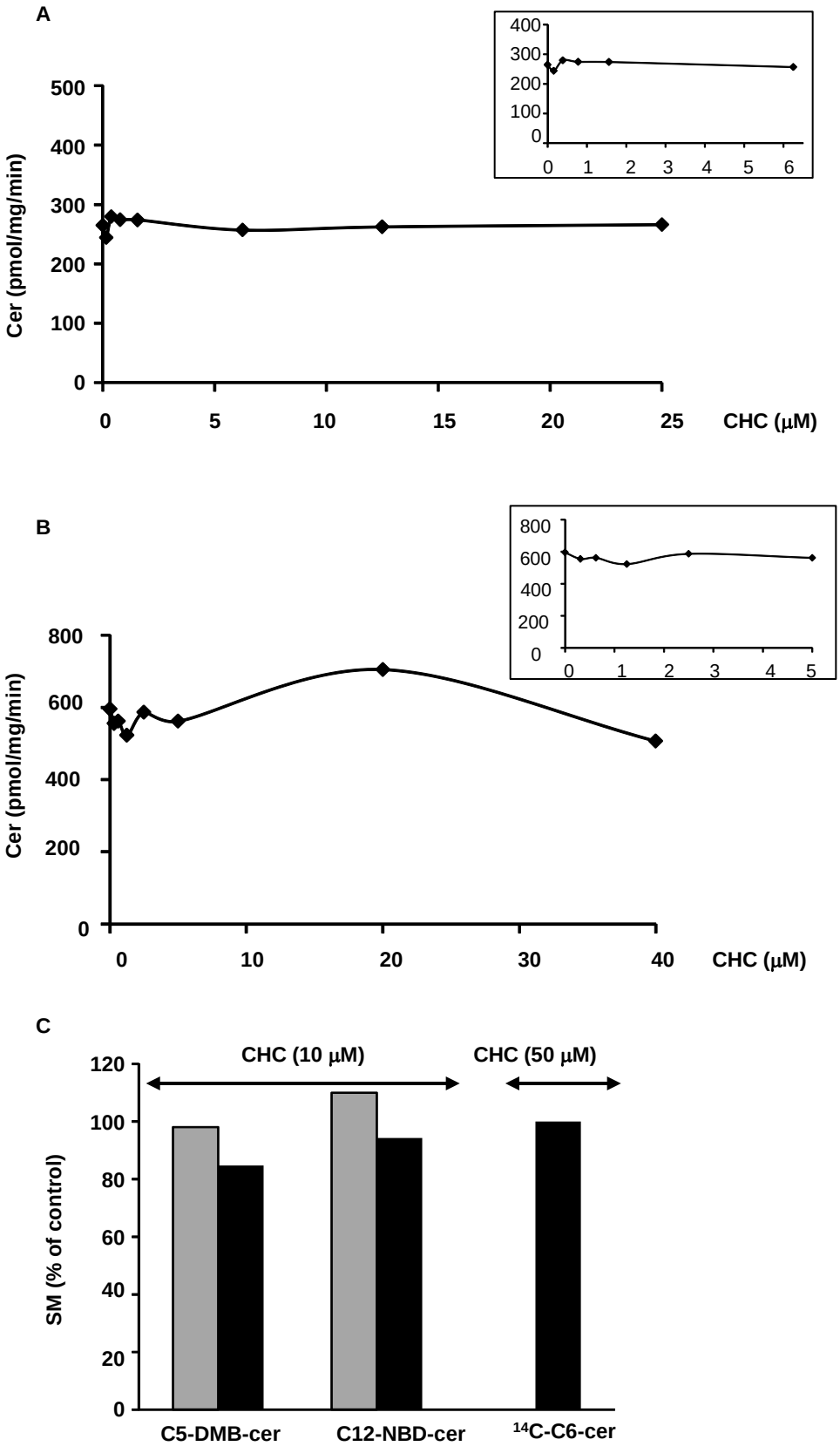
Suppl. Fig. S5: Mass spectra of Limonoid HC after cell incubation: CHO cells were incubated with 10 μM HC as in Fig S4B. Then limonoids were extracted and analyzed by LCMS (upper spectrum **A, B, C**) and MS-MS (lower spectrum **A, B, C**). The MS-MS analysis indicated that the precursor ion m/z 485.5,

$[M+H]^+$ was originated from intact HC molecule as found in the standard solution (**A**) and after 1 h (**B**) and 24 h (**C**) incubation with cells.

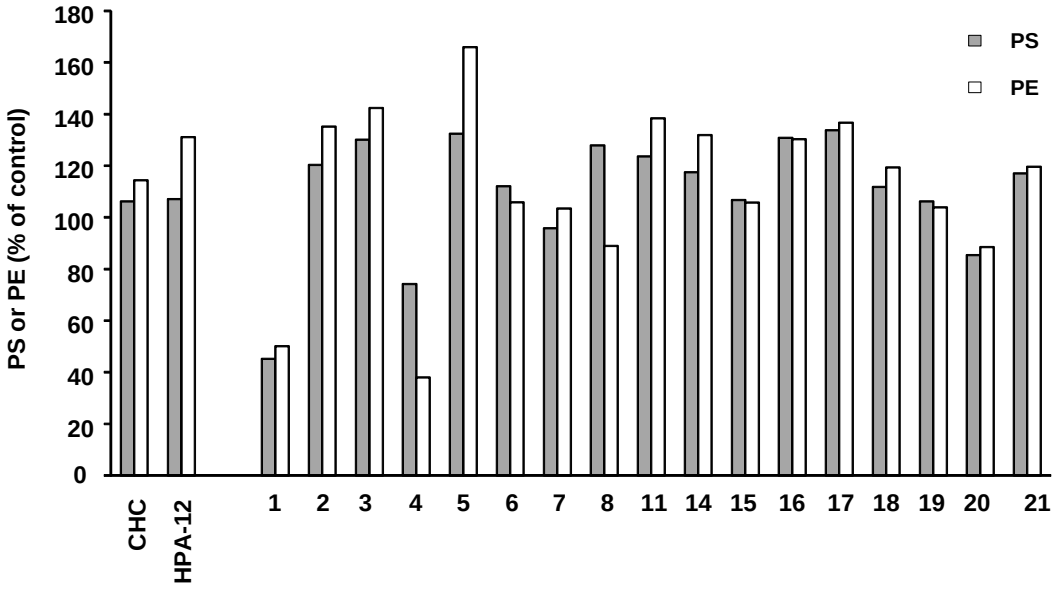
References

1. Yasuda, S., Kitagawa, H., Ueno, M., Ishitani, H., Fukasawa, M., Nishijima, M., Kobayashi, S., and Hanada, K. (2001) *J Biol Chem* **276**, 43994-44002
2. Kobayashi, T., Storrie, B., Simons, K., and Dotti, C. G. (1992) *Nature* **359**, 647-650
3. Takahashi, M., Murate, M., Fukuda, M., Sato, S. B., Ohta, A., and Kobayashi, T. (2007) *Mol. Biol. Cell* **18**, 2667-2677
4. Koval, M., and Pagano, R. E. (1989) *J Cell Biol* **108**, 2169-2181
5. Lahiri, S., Lee, H., Mesicek, J., Fuks, Z., Haimovitz-Friedman, A., Kolesnick, R. N., and Futerman, A. H. (2007) *FEBS Letters* **581**, 5289-5294
6. Lahiri, S., Park, H., Laviad, E. L., Lu, X., Bittman, R., and Futerman, A. H. (2009) *J Biol Chem* **284**, 16090-16098
7. Bligh, E. G., and Dyer, W. J. (1959) *Can J Biochem Physiol* **37**, 911-917
8. Yao, Z. M., and Vance, D. E. (1988) *J Biol Chem* **263**, 2998 - 3004
9. Hanada, K., Horii, M., and Akamatsu, Y. (1991) *Biochim Biophys Acta* **1086**, 151-156
10. Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, K. J., and Lippincott-Schwartz, J. (1997) *Nature* **389**, 81-85
11. Sciaky, N., Presley, J., Smith, C., Zaal, K. J., Cole, N., Moreira, J. E., Terasaki, M., Siggia, E., and Lippincott-Schwartz, J. (1997) *J Cell Biol* **139**, 1137-1155
12. Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H., Jr. (1991) *J Biol Chem* **266**, 14486-14490
13. Owens, K. (1966) *Biochem J* **100**, 354-361
14. Chalvardjian, A., and Rudnicki, E. (1970) *Anal Biochem* **36**, 225-226
15. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J Biol Chem* **226**, 497-509
16. Hayakawa, T., Makino, A., Michaud, S., Lagarde, M., Douteau, A., Ito, K., Hirai, M., and Kobayashi, T. (2007) *Membrane* **32**, 221-228
17. Hayakawa, T., Hirano, Y., Makino, A., Michaud, S., Lagarde, M., Pageaux, J. F., Doutheau, A., Ito, K., Fujisawa, T., Takahashi, H., and Kobayashi, T. (2006) *Biochemistry* **45**, 9198-9209

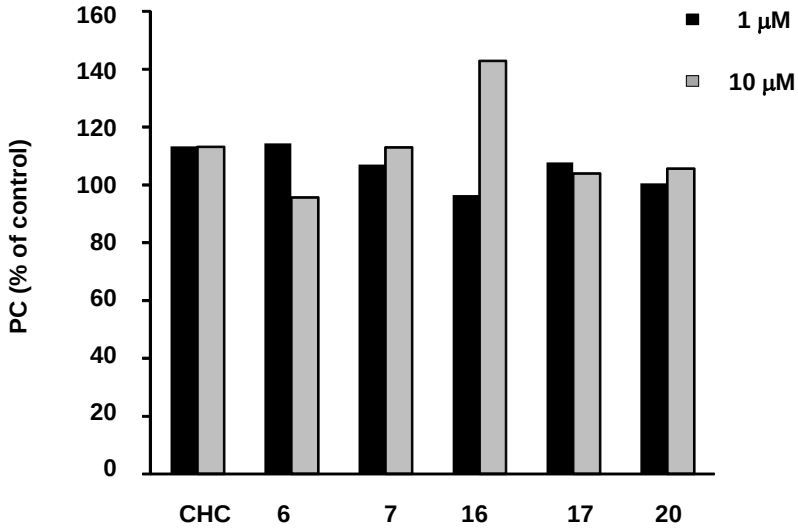

A
B
C
D
E
F


A
Cer (pmol/mg/min)
CHC (μM)
B
Cer (pmol/mg/min)
CHC (μM)
C
SM (% of control)
CHC (10 μM)
CHC (50 μM)
C5-DMB-cer
C12-NBD-cer
14C-C6-cer

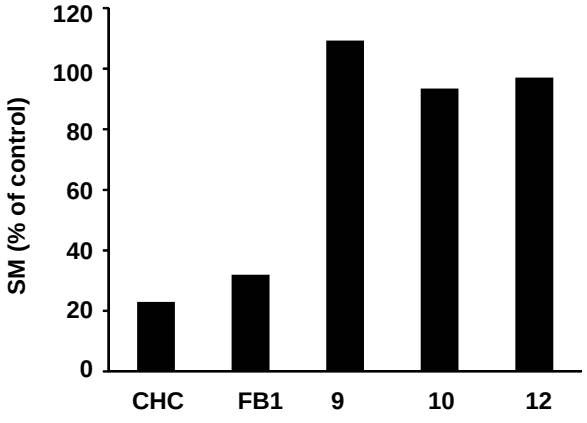
A


PS or PE (% of control)
180
160
140
120
100
80
60
40
20
0
PS
PE
CHC
HPA-12
1
2
3
4
5
6
7
8
11
14
15
16
17
18
19
20
21

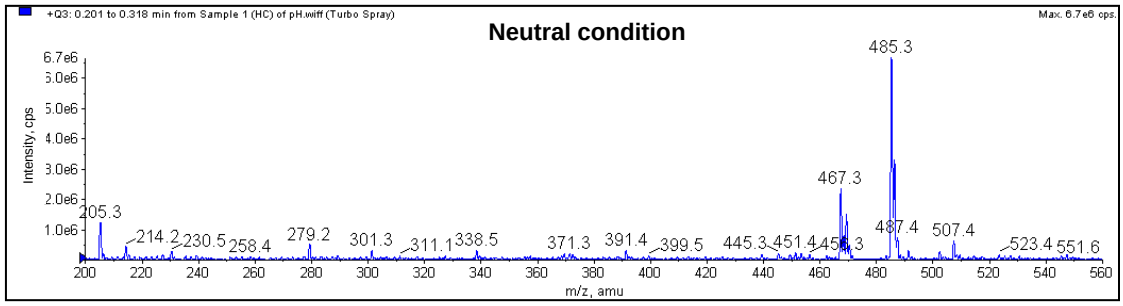
B

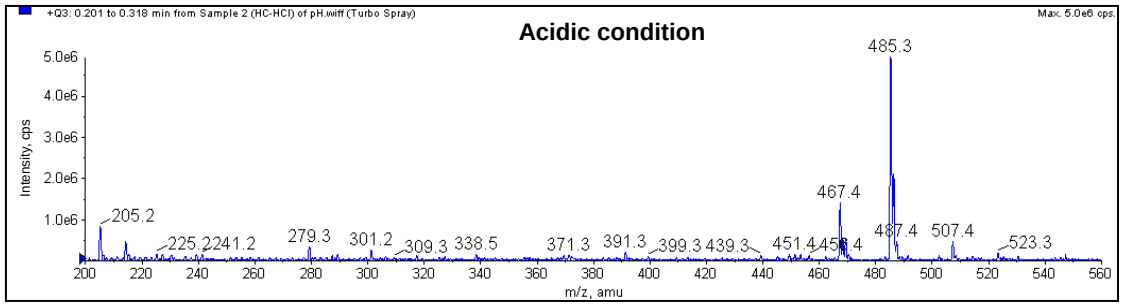

PC (% of control)
160
140
120
100
80
60
40
20
0
1 μM
10 μM
CHC
6
7
16
17
20

C

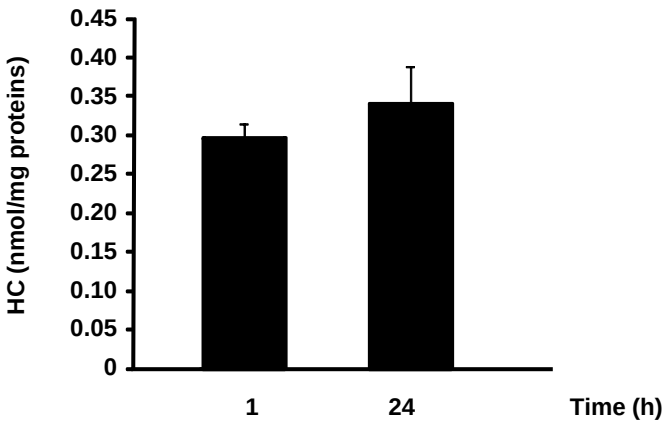

SM (% of control)
120
100
80
60
40
20
0
CHC
FB1
9
10
12

A

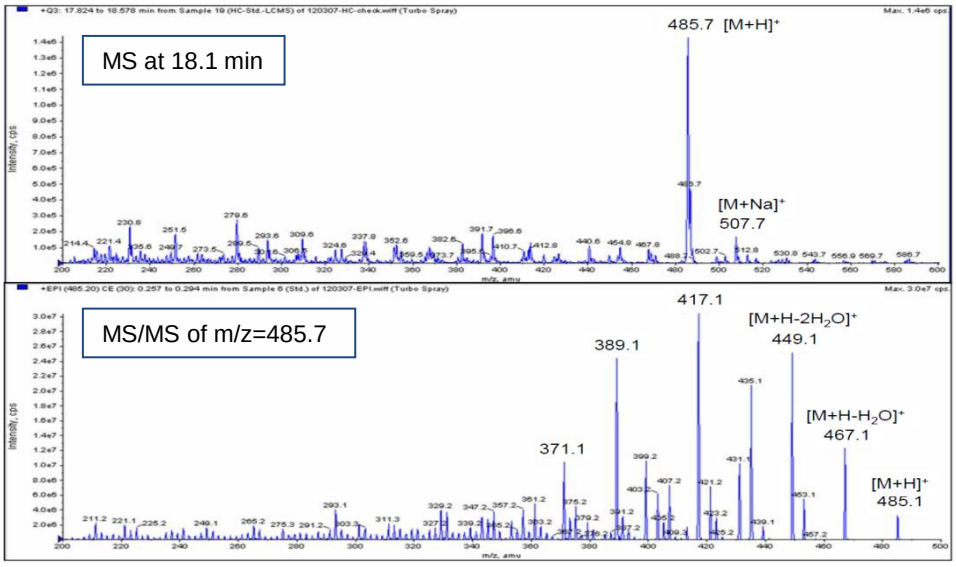

+Q3: 0.201 to 0.318 min from Sample 1 (HC) of pH.wiff (Turbo Spray)
Max. 6.7e6 cps.
Neutral condition
Intensity, cps
m/z, amu
485.3
467.3
205.3
487.4
507.4
214.2
230.5
258.4
279.2
301.3
311.1
338.5
371.3
391.4
399.5
445.3
523.4
551.6


+Q3: 0.201 to 0.318 min from Sample 2 (HC-HCl) of pH.wiff (Turbo Spray)
Max. 5.0e6 cps.
Acidic condition
Intensity, cps
m/z, amu
485.3
467.4
205.2
487.4
507.4
225.2
241.2
279.3
301.2
309.3
338.5
371.3
391.3
399.3
439.3
451.4
523.3

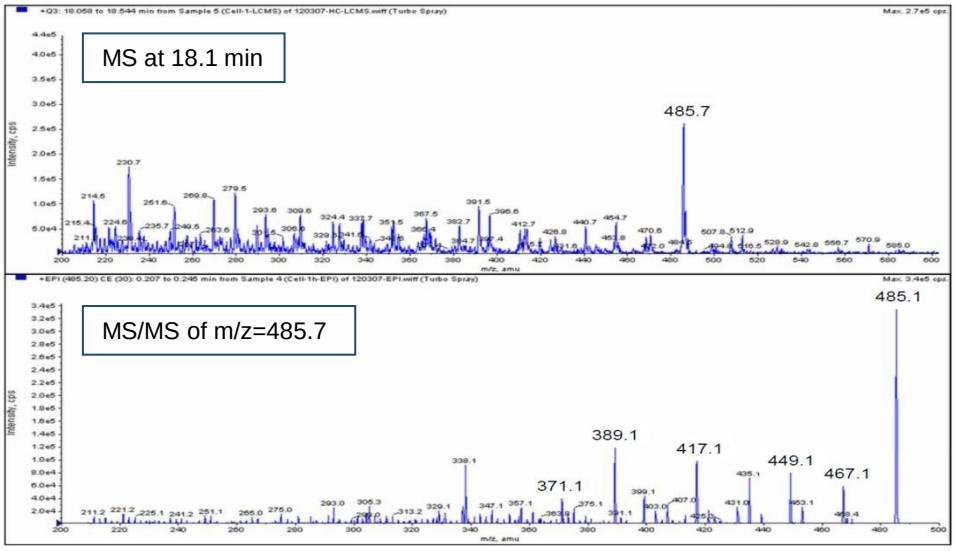
B


HC (nmol/mg proteins)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1
24
Time (h)

A- Standard HC



B- Cell 1h



C- Cell 24h